Molecular and Experimental Basis for COX Inhibition in Cancer

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Introduction

Experimental, epidemiological, and randomized clinical studies have led to accumulate numerous pieces of evidence suggesting that the pharmacological inhibition of cyclooxygenase (COX)-isozymes by nonsteroidal anti-inflammatory drugs (NSAIDs), including aspirin, has a protective effect against tumor development [1]. NSAIDs are a group of chemically heterogeneous molecules which act by inhibiting the synthesis of prostanoids through the prevention of arachidonic acid (AA) binding in the active site of COX-1 and COX-2. An important prostanoid involved in tumorigenesis is prostaglandin (PG)E_2. Enhanced biosynthesis of PGE_2 has been detected in various types of human malignancies including colorectal, lung, breast, and head and neck cancer and is often associated with a poor prognosis [2–5]. This prostanoid binds to and activates G-protein-coupled prostaglandin E1-4 receptors (EP1-4) and exerts a profound influence over the adhesive, migratory, and invasive behavior of cells during the development and progression of cancer [6]. Moreover, PGE_2 may contribute to the formation, maintenance, and expansion of cancer stem cells (CSCs), which have the capacity for self-renewal, differentiation, and resistance to cytotoxic agents [7]. Finally, enhanced PGE_2 production can generate an immunosuppressive microenvironment that allows advantages for tumor formation and progression [8].

The role of COX-2-dependent PGE_2 in intestinal tumorigenesis is strongly supported in humans by the results of clinical studies showing that selective COX-2 inhibitors (named coxibs) cause the reduction of polyp number and size in familial adenomatous polyposis (FAP) patients [9] and prevent polyp reoccurrence in patients with sporadic adenomas [10–12]. However, the role of prostanoids in colorectal carcinogenesis is more complex in the light of the findings that the antiplatelet agent low-dose aspirin may cause similar effects, in the same clinical conditions [13]. In fact, it is unlike that low-dose aspirin acts through an inhibitory effect on COX-2 activity expressed in colorectal adenomas; in contrast, several lines of evidence suggest that the drug acts by affecting COX-1-dependent prostanoid biosynthesis, mainly thromboxane (TX)A_2, in platelets.

The efficacy of the antiplatelet agent low-dose aspirin has opened new avenues in the understanding of the mechanisms involved in colorectal cancer (CRC) development and progression

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and in the development of novel therapeutic anticancer strategies.

The aim of the present chapter is to enlighten the biology and functions of COX-1 and COX-2 and the derived prostanoids PGE_2 and TXA_2 in relation to tumorigenesis (mainly in the colorectum) and cancer metastasis. Moreover, we will characterize the different mechanisms of action of NSAIDs selective for COX-2 (coxibs) versus low-dose aspirin in regard to their anticancer effects.

Prostanoid Signaling in Cancer and Metastasis

The results of clinical studies showing the chemopreventive effect of NSAIDs in cancer [1] support the notion that COX-derived prostanoids play an important role in tumor development and progression. Here, a detailed description of the biosynthesis and activities of prostanoids (Fig. 12.1) and their implication in intestinal tumorigenesis is reported.

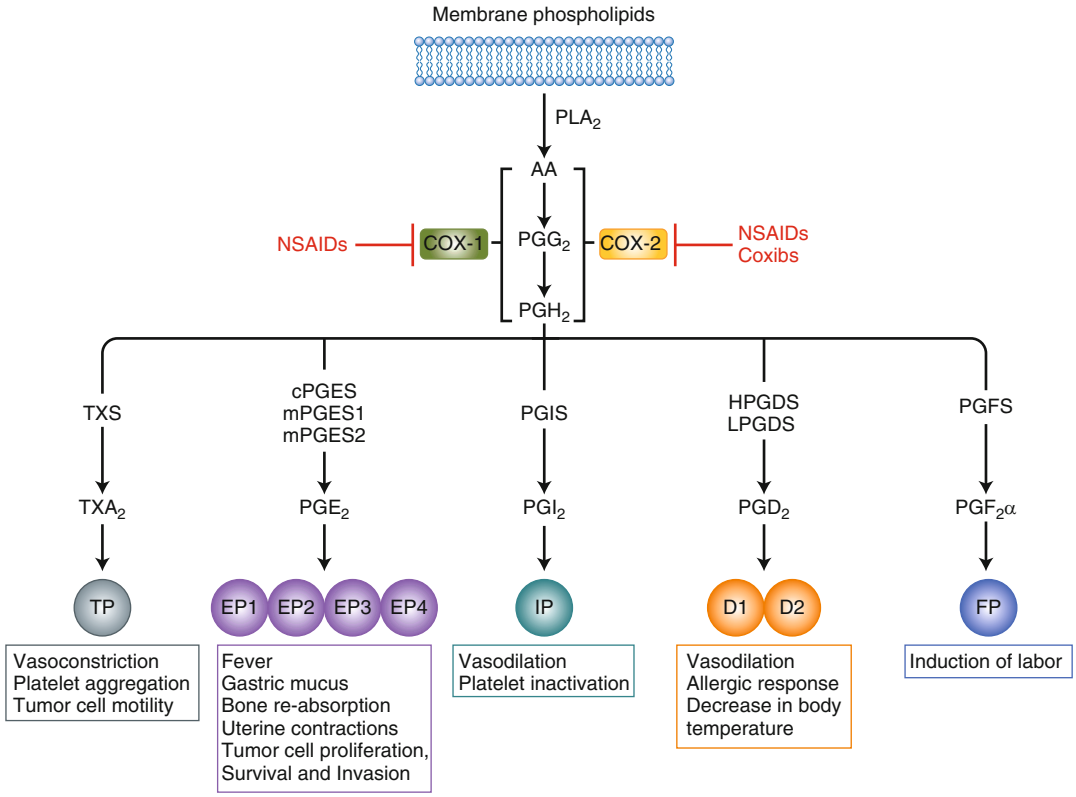

Fig. 12.1 Prostanoid biosynthesis and main functions in physiological and pathological settings. Arachidonic acid can be metabolized through the cyclooxygenase isozymes: COX-1 and COX-2. The derived prostaglandins (PGE_2, PGI_2, PGD_2, $PGF_{2\alpha}$) and TXA_2 exert a variety of biological functions through their respective cognate receptors. *AA* arachidonic acid, *COX* cyclooxygenase, *PG* prostaglandin, *PLA_2* phospholipase A2, *TXA_2* thromboxane A_2, *TXS* thromboxane synthase, *cPGES* cytosolic PGE_2 synthase, *mPGES* microsomal PGE_2 synthase, *PGIS* PGI_2 synthase, *H-PGDS* hematopoietic PGD_2 synthase, *L-PGDS* lipocalin PGD_2 synthase, *PGFS* $PGF_{2\alpha}$ synthase, *TP* TXA_2 receptor, *EP* PGE_2 receptor, *IP* PGI_2 receptor, *DP* PGD_2 receptor *FP*, PGF_{2a} receptor, *NSAIDs* nonsteroidal anti-inflammatory drugs, *Coxibs* COX-2 selective inhibitors

Prostanoid Generation: The Activity of COX Isozymes

COX-1 and COX-2 (also known as PGG/H synthase-1 and synthase-2, respectively) are homodimers of 576 and 581 amino acids, respectively [14]. Each subunit of the dimer contains the cyclooxygenase and peroxidase active sites which contribute to catalyze the rate-limiting step of prostanoid biosynthesis, i.e., the production of PGH_2 from AA, which is released from membrane phospholipids by phospholipases (PL), mainly cytosolic (c) PLA_2 upon cellular activation [15]. PGH_2 is then transformed to prostanoids by the activity of different terminal synthases. Prostanoids are a family of bioactive lipids which comprises PGE_2, $PGF_{2\alpha}$, PGD_2, prostacyclin (PGI_2), and TXA_2 (Fig. 12.1).

Despite COX-1 and COX-2 produce the same prostanoids, their cellular levels are influenced by the extent of expression of the corresponding genes. The genes of COX-1 (*PTGS1*) and COX-2 (*PTGS2*) have a different regulation: (1) *PTGS2* is an immediate early response gene that is normally absent from most cells but is highly induced at sites of inflammation and during tumor progression [16], while (2) *PTGS1* is a housekeeping enzyme responsible for maintaining basal prostanoid levels that are important for tissue homeostasis [16].

Prostanoids play important roles in many physiological and pathophysiologic processes, including inflammation and its resolution, erosion of cartilage and juxta-articular bone, gastrointestinal (GI) cytoprotection and ulceration, angiogenesis and cancer, hemostasis and thrombosis, renal hemodynamics and progression of kidney disease, and atheroprotection and progression of atherosclerosis [17–19]. COXs act as oxidase enzymes that first peroxidate AA to form the hydroperoxyendoperoxide PGG_2, which links two oxygen molecules across carbons 9 and 1. As second coordinate enzymatic function, COXs reduce a hydroperoxy group at carbon 15 of PGG_2 to form the intermediate product PGH_2 which serves as substrate for a variety of PG synthases involved in prostanoid biosynthesis [14] (Fig. 12.1). The generation of PGE_2 is catalyzed by three different synthases: a cytosolic PGE synthase (cPGES) and two membrane-bound PGESs, i.e., mPGES-1 and mPGES-2 [20]. Whereas cPGES and mPGES-2 are constitutive enzymes, mPGES-1 is encoded by an inducible gene. It is thought that the coordinated expression of COX-2 and mPGES-1 is responsible for enhanced biosynthesis of PGE_2 which occurs in inflammation and cancer [20]. The biosynthesis of PGD_2 is regulated by the activity of two PGD synthases, lipocalin (L-PGDS) and hematopoietic (H-PGDS) [21]. Finally, the biosynthesis of TXA_2 and PGI_2 involves the activity of TX-synthase (TXS) and PGI-synthase (PGIS), respectively [19] (Fig. 12.1).

Prostanoids are second messengers which can cross the cell membrane, diffuse through the extracellular space, and interact with high-affinity G-protein-coupled receptors (GPCRs) on the same cell or in neighboring cells. The prostanoid receptor family consists of eight rhodopsin-like (class A) GPCRs, each being the product of an individual gene: DP1 (for PGD_2); EP1, EP2, EP3, and EP4 (for PGE_2); FP (for $PGF_{2\alpha}$); IP (for PGI_2); and TP (for TXA_2) [14]. The prostanoid receptor named chemoattractant receptor-homologous molecule expressed on T-helper type 2 cells (CRTH2) or DP2, characterized by a higher sequence homology with other leukocyte chemoattractant receptors than prostanoid receptors [22], also acts as receptor for PGD_2.

The specific action of the different prostanoids in a particular type of tissue predominantly depends on the cell-type-specific expression of their receptors as well as prostanoid production. In addition to their biosynthesis, the extracellular levels of prostanoids also depend on a carrier-mediated transport process, as well as inactivation in the cytoplasm. These processes are regulated by prostaglandin transporter (PGT, an influx transporter), multidrug resistance-associated protein 4 (MRP4, an efflux transporter), and hydroxyprostaglandin dehydrogenase 15-(NAD) (HPGD, also known

15-PGDH). For example, PGE_2 and $PGF_{2\alpha}$ are rapidly metabolized in vivo by 15-PGDH to stable 13,14-dihydro-15-keto-PGE_2 (PGEM) and 13,14-dihydro-15-keto-PGF2α, respectively.

Involvement of PGE_2-EP Signaling in Tumorigenesis

Among prostanoids, pro-inflammatory PGE_2 has a predominant role in promoting tumor growth [23, 2–3]. PGE_2 is the most abundant prostaglandin that is found in various human malignancies, including colon, lung, breast, and head and neck cancer, and is often associated with a poor prognosis [4, 5]. By contrast, 15-PGDH is highly expressed in normal tissues but is ubiquitously lacking in human colon, gastric, lung, and breast cancer [24–27]. The lack of 15-PGDH expression in these tumors results in increased endogenous PGE_2 levels.

The role of PGE_2 in colon cancer progression arose from studies with the $APC^{Min/+}$mouse model of intestinal neoplasia. This genetic animal model for FAP maintains inactivating mutations in the adenomatous polyposis coli (*APC*) gene [28]. Treatment of these animals with PGE_2 promoted a dramatic increase in small and large intestinal tumor burden [23]. Moreover, studies in humans revealed that adenoma regression was more effective when PGE_2 tissue levels were profoundly inhibited by treatment with NSAIDs [29]. The activation of the canonical Wnt pathway in the colonic epithelium is a key event in polyp formation, and this event is associated with the upregulation of several genes involved in tumor development and progression [30]. Among them, overexpression of COX-2 plays a central role in intestinal tumorigenesis. In fact, elevated levels of COX-2-derived PGE_2 are associated with (1) resistance to apoptosis, through the upregulation of the antiapoptotic protein Bcl-2 and the induction of nuclear factor-kB (NF-kB) transcriptional activity [23], (2) stimulation of cell proliferation, (3) stimulation of cell migration, and (4) angiogenesis [23] (Fig. 12.2a).

In addition, PGE_2 may contribute to the formation, maintenance, and expansion of CSCs, by activating NF-kB, via EP4-PI3K and EP4-mitogen-activated protein kinase signaling, and promotes the formation of liver metastases in mice [7] (Fig. 12.2a). Enhanced PGE_2 production, which occurs in chronic inflammation, can generate an immunosuppressive microenvironment that allows advantages for tumor formation and progression [8]. The well-recognized role of PGE_2 during tumor promotion coupled with findings demonstrating that long-term use of NSAIDs may be associated with GI toxicity [31] and increased risk of adverse cardiovascular (CV) events [32, 33], provided the rationale for the identification of novel enzymatic targets within the AA pathway, including the PGE_2 terminal synthases [34].

Role of mPGES1 in Tumorigenesis

mPGES-1 is a member of the membrane-associated proteins involved in eicosanoid and glutathione metabolism (MAPEG) superfamily, showing significant homology with other MAPEG superfamily proteins. mPGES-1 is expressed at minimal levels in most normal tissues, although abundant and constitutive expression is detected in a limited number of organs, such as the lung, kidney, and reproductive organs. mPGES-1 is also induced by cytokines and various growth factors [35].

mPGES-1 is functionally coupled with COX-2, and its expression is often concomitantly induced with COX-2 overexpression, thus contributing to the efficient generation of PGE_2 during inflammation [36]. However, studies using diverse stimuli provided evidence that COX-2 and mPGES-1 can be independently regulated [37]. This observation suggested the possibility that the pharmacological targeting of mPGES-1 may result in the suppression of PGE_2 production by mechanisms that circumvent the CV toxicity associated with inhibition of COX-2 activity by NSAIDs, both traditional(t) and coxibs [38].

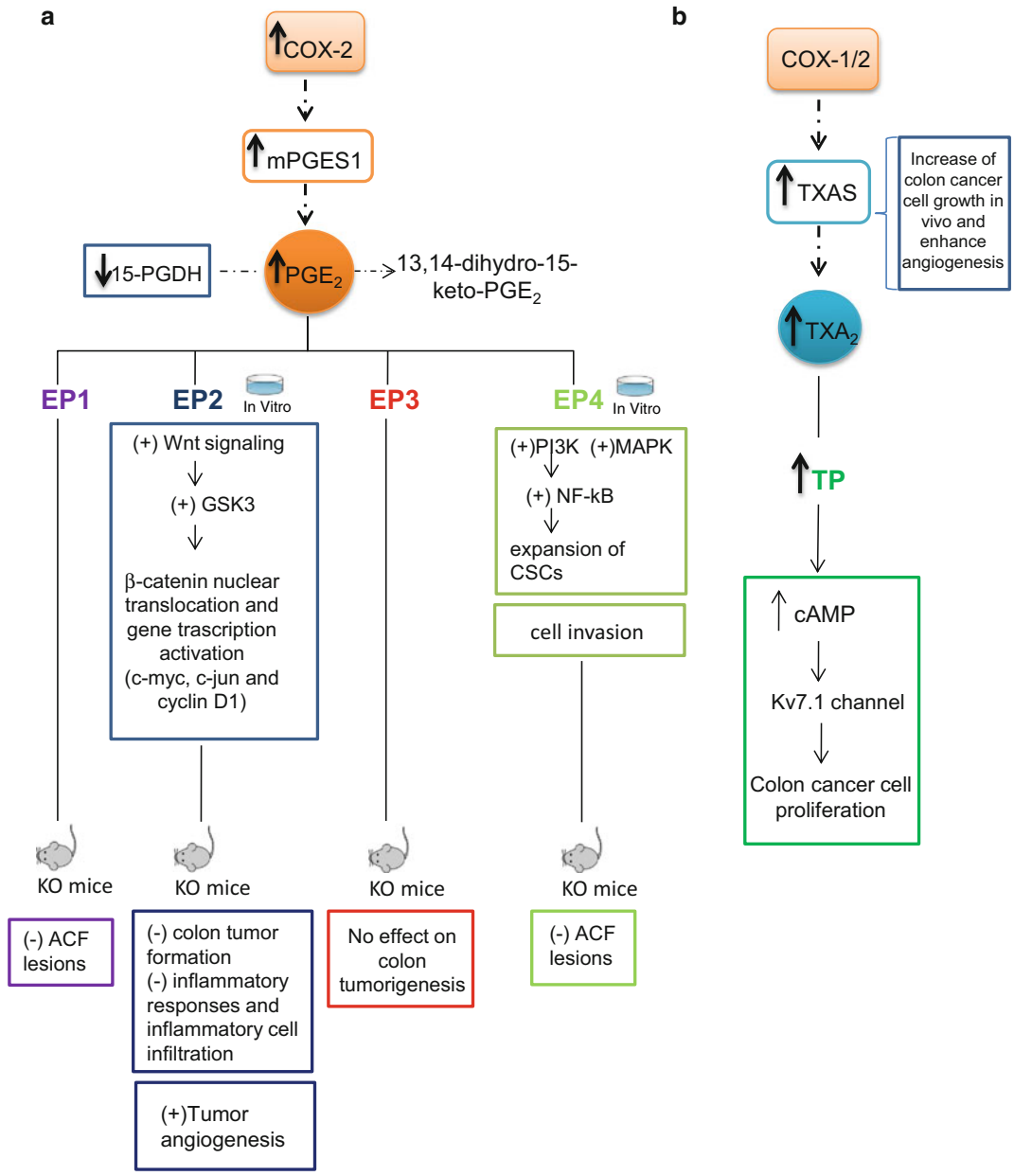

Fig. 12.2 PGE_2-EP (**a**) and TXA_2-TP (**b**) signaling in intestinal tumorigenesis. In intestinal tumorigenesis, the increased levels of PGE_2 derived from COX-2 and mPGES1 overexpression couples with the reduction of the PGE_2-degrading enzyme, 15-PGDH (**a**). In addition, in this setting, the increase of TXAS expression leads to the enhanced biosynthesis of TXA_2 (**b**). The interaction of PGE_2 and TXA_2 with their receptors exerts a protumorigenic effect through several mechanisms. In addition, the implication of EP receptors in tumorigenesis was demonstrated by several in vivo studies using specific knockout mice. *COX* cyclooxygenase, *PG* prostaglandin, *TXA_2* thromboxane A_2, *TXAS* thromboxane synthase, *mPGES* microsomal PGE_2 synthase, *TP* TXA_2 receptor, *EP* PGE_2 receptor, *KO* knockout, *15-PGDH* 15-hydroxyprostaglandin dehydrogenase, *GSK* glycogen synthase kinase 3, *ACF* aberrant crypt foci, *PI3K* phosphatidylinositol 3-kinase, *MAPK* mitogen-activated protein kinase, *NF-kB* nuclear factor kappa-light-chain-enhancer of activated B cells, *CSC* cancer stem cell, *cAMP* cyclic adenosine monophosphate, *Kv channel* voltage-gated K+ channel

On the basis of evidence from cell culture studies, several in vivo studies have been performed to address the impact of mPGES-1 targeting on colon tumorigenesis, but the results of these studies are conflicting [39]. In particular, mPGES-1 knockout (KO) mice in a mutant *APC* background showed a significant reduction in the number and size of intestinal tumors. In contrast, Elander et al. reported that genetic deletion of mPGES-1 resulted in accelerated intestinal tumorigenesis in $APC^{Min/+}$ mice [40]. Environmental factors (i.e., *Helicobacter pylori* infection) may represent a possible explanation of these different responses along with genetic differences in the mouse models used. mPGES-1 KO mice were also protected against azoxymethane (AOM)-induced colon cancer with reduced number of aberrant crypt foci (ACF), putative preneoplastic lesions of the colon, and up to 90 % decrease in tumor load in the distal colon [41]. Genetic deletion of mPGES-1 in a HER2 receptor-driven breast cancer mouse model also showed reduced number of larger tumors, in addition to suppression of angiogenesis in mammary glands [42]. In a study using xenografts from prostate cancer cells (RM9), Takahashi et al. showed that the number of lung metastases and tumor in the lung was reduced in mice treated with the coxib celecoxib. The same effect was obtained in mPGES-1 KO mice. The reduction in lung metastases was also coupled to a decrease in angiogenesis in the lung tissue [43]. It has been shown that the tumor growth in mouse xenografts using a lung cancer cell line (A549) and a prostate cancer cell line (DU145) with mPGES-1 knockdown (KD) was considerably slowed compared to cells with endogenous mPGES-1 expression [44]. Altogether these results have successfully demonstrated that the selective inhibition of mPGES-1 is a possible way to avoid general and detrimental downregulation of prostaglandins resulting from COX inhibition, but there is no selective mPGES-1 inhibitors on the market. Recent progress in the field has generated inhibitors active against both human and murine enzymes which will be an important tool to evaluate in animal studies the antitumor effect as well as to rule out toxic side effects of this pharmacological approach. Moreover, the possible shunting to other prostanoids needs to be investigated thoroughly, and the pharmacological inhibition of mPGES-1 also needs to be carefully compared to COX-1 and COX-2 selective inhibitors both in terms of antitumor efficacy and side effects.

Role of EP Receptors in Tumorigenesis

PGE_2 acts by the activation of four subtypes of receptor, known as EP1, EP2, EP3, and EP4 (Figs. 12.1 and 12.2a). These receptors belong to three clusters within the G-protein-coupled receptor superfamily of seven transmembrane-spanning proteins. EP2 and EP4 can form one cluster, transferring the signaling through increased cyclic AMP (cAMP) mediated by Gαs. EP3 is coupled to Gi and causes the decrease of cAMP formation. EP1 can increase the intracellular calcium through Gq [14]. There are several lines of evidence indicating the involvement of EP receptors in tumor progression. For example, PGE_2 regulates tumor growth by stimulating angiogenesis via EP2 [45], and EP4 is overexpressed in epithelial cancers and colorectal adenomas, mediating the functions of PGE_2 in cancer cell invasion and metastasis formation [46–49]. However, by binding EP receptors on the cell surface, PGE_2 not only can activate the downstream G-proteins but also can indirectly trigger Wnt signaling, peroxisome proliferator-activated receptor (PPAR)-δ, and epithelial growth factor receptor (EGFR) pathway. Moreover, PGE_2 can cause nuclear localization of β-catenin and increase the transcriptional targets, such as c-myc, c-jun, and cyclin D1, of Wnt signaling. The aberrant activation of Wnt pathway is thought to be relative to the initiation of various types of epithelial tumors, including CRC. In a study published in 2005 [47], it was demonstrated that PGE_2-mediated stimulation of EP2 receptors led to a direct association of the α-subunit of the regulator G-protein signaling and axin. Further, this binding causes inactivation of

GSK-3β, the downstream effector in Wnt pathway, and results in the accumulation of β-catenin in the nucleus [47].

The recent establishment of mice lacking the genes encoding EP receptors [50–52] has enhanced our understanding of the involvement of PGE_2 and its receptors in the development of colon cancer (Fig. 12.2a). Watanabe and colleagues [51] examined the development of ACFs in two strains in EP receptor (EP1 and EP3, respectively) KO mice, by treatment with the colon carcinogen AOM. The formation of ACFs was decreased only in the EP1 KO mice, and the administration of the selective EP1 antagonists, ONO-8711 [48] and ONO-8713 [51], to AOM-treated wild-type mice also resulted in a dose-dependent reduction of ACF formation. The same approach using EP3 KO mice indicated that the deficiency of EP3 receptor has no effect on colon carcinogenesis [49]. These results strongly suggest that PGE_2 contributes to colon carcinogenesis to some extent through its action at the EP1 receptor and EP1 antagonists may be good candidates as chemopreventive agents for colon cancer. In the study performed by Mutoh et al., the authors reported the development of ACFs in mice lacking EP2 or EP4 receptors and observed that only the deletion of EP4 reduced the formation of ACF lesions [49]. These data were confirmed by using the EP4-selective antagonist on the formation of colon ACFs induced by AOM in C57BL/6 mice and on the development of intestinal polyps in $APC^{Min/+}$ mice [49]. Recently, Ma and colleagues examined actions of PGE_2 in tumor microenvironment in colon tumorigenesis by using a model of colitis-associated cancer (CAC) in KO mice deficient for EP1, EP2, or EP3 receptors [52]. Among the different murine models, only EP2 KO mice showed significant suppression of colon tumor formation with reduced inflammatory responses and inflammatory cell infiltration. In fact, the authors found that EP2 in neutrophils and tumor-associated fibroblast promotes colon tumorigenesis by amplifying inflammation and shaping tumor microenvironment [52].

Involvement of TXA_2-TP Signaling in Tumorigenesis

TXA_2 is a potent vasoconstrictor, mitogen, and platelet activator [53–55], and it may be also implicated in cellular hypertrophy [56]. TXA_2 acts via the binding to the TXA_2 receptor (TP), a member of the seven transmembrane G-protein-coupled receptor superfamily. TPs are widely distributed in different organs, and they are localized on both cell membranes and intracellular structures. Two alternatively spliced variants of human TP have been described [57], and they differ in amino acids (aa) sequence at the C-terminal tail of the receptor. The original placenta-derived clone of 343 aa receptor has been designated as TPα, and a 407 aa splice variant cloned from endothelium is designated as TPβ. TP mRNAs are widely expressed in the lung, liver, kidney, heart, uterus, and vascular cells [58]. In these organs, TPα is the dominant isoform. Both TPα and TPβ couple via Gq, G11, and G12/13 to activate PLC-dependent inositol phosphate generation and elevate intracellular calcium [58], leading to vasoconstriction and platelet aggregation [59, 60]. In recent years, several studies have indicated functional roles for both TXS and TP in cancer progression in different organs such as the prostate, breast, lung, brain, bladder, and colon [61]. TXS and TXA_2 biosynthesis are increased in colon cancer and cause detrimental effects by promoting TP signaling [62] (Fig. 12.2b). The involvement of TXS in colon tumorigenesis was investigated by Pradono and colleagues [62]; in this study, the gene transfer of TXS increased colon cancer cell growth in vivo and enhanced angiogenesis. Strong evidence of a role for TXS in colorectal carcinoma was provided when its marked overexpression was observed in colorectal tumors of different grades compared to the paired normal tissues. The same study found increased expression of TXS in colon cancer cell lines and showed that abrogation of TXA_2 signaling with TXS inhibitors and TXS anti-sense as well as TP antagonists reduced proliferation of the CRC cell lines [63, 64]. The expression levels

of TP mRNA were assessed in 62 tumors and adjacent normal colon tissues: TP was expressed at higher levels in tumors compared to normal tissues but displayed lower levels in cultured CRC cell lines (HT-29 and HCA-7) [63]. The mechanism of TXA_2-induced cancer cell proliferation has been studied by Shimizu and collaborators [65] who found that a voltage-gated K+ channel, Kv7.1, is involved in the TXA_2-induced colon cancer cell proliferation and that it is upregulated by the TXA_2 receptor-mediated cAMP pathway [65] (Fig. 12.2b). In addition, in patients with FAP, which is an inherited disorder characterized by cancer of the large intestine (colon) and rectum, enhanced generation of TXB_2 in vivo was found by the assessment of its urinary enzymatic metabolite, 11-dehydro-TXB_2 [66]. In these patients, the levels of TXM were unaffected by the administration of celecoxib suggesting the involvement of a COX-1-dependent pathway, presumably from platelets. Furthermore, Sciulli et al. [67] have reported that enhanced systemic biosynthesis of TXA_2 was detected in patients with CRC and it is mainly from platelet COX-1 since it is reduced by the administration of low-dose aspirin.

NSAIDs and Cancer Prevention: Clinical Evidences

The central role of COX isozymes in human tumorigenesis is supported by the efficacy of tNSAIDs and coxibs in protecting against some cancers, particularly of the lower GI tract [1]. Epidemiologic (nonrandomized) studies have found that long-term users of aspirin or other NSAIDs have a lower risk of colorectal adenomatous polyps and CRC than nonusers, although one study has not [1]. Randomized clinical trials (RCTs) have confirmed that two NSAIDs, the prodrug sulindac [29, 68, 69] and the selective COX-2 inhibitor celecoxib [9], effectively inhibit the growth of adenomatous polyps and cause regression of existing polyps in patients with FAP. Recently, in a prospective, observational study, patients with stage III colon cancer that were enrolled in an adjuvant chemotherapy trial showed both significantly increased "event-free" and "overall" survival after consistent use of aspirin or COX-2 selective drugs (celecoxib or rofecoxib) during and after chemotherapy (according to questionnaires) [70]. Despite these positive findings, the main limit to the use of NSAIDs is that they share both beneficial and adverse effects due to the same mechanism of action, i.e., the inhibition of COX activity [71]. Their beneficial therapeutic use as anti-inflammatory and analgesic agents is associated with increased risk of clinically relevant GI side effects, i.e., GI bleeding, perforation and obstruction [31, 32], and CV side effects, including an increased risk of nonfatal myocardial infarction [32, 33, 72, 73]. NSAIDs injure the GI tract by causing topical injury to the mucosa and by systemic effects associated with mucosal prostanoid depletion derived from COX inhibition. CV toxicity is probably linked to the inhibition of COX-2-derived PGI_2 in vascular endothelial cells in the absence of an almost complete and persistent inhibition of platelet COX-1-derived TXA_2 [73]. Thus, the efficacy and safety of long-term NSAID prophylaxis against colorectal or other cancers remain unproven, and fundamental questions remain about their safety, efficacy, mechanisms of action, optimal treatment regimens, and contraindications for preventive therapy. Concerns about NSAID-associated CV toxicity have also refocused attention on the chemopreventive properties of aspirin.

Aspirin as Chemopreventive Agent

The recent results of the analysis of RCTs with aspirin by Rothwell and colleagues have fueled a renewed interest in performing studies to elucidate COX-dependent and COX-independent mechanisms of cancer prevention. The lines of evidence supporting the chemopreventive effect of aspirin against cancer mainly refer to GI tract tumors [1, 74, 75]. In particular, these evidences are derived from a large number of case-control and cohort studies reporting the association

between aspirin administration and reduced risk of different types of cancer, with largest effects on risk of GI cancers [76]. Moreover, four randomized, placebo-controlled trials in subjects with sporadic colorectal adenomas and their meta-analysis [77] demonstrated reduced risk of recurrence in aspirin-treated subjects, and a placebo-controlled, RCT in patients with Lynch Syndrome (LS, hereditary nonpolyposis colon cancer) showed that aspirin reduced cancer incidence during long-term follow-up [78] but not during the scheduled treatment phase of the trial [79]. Finally, a post hoc, individual patient data meta-analysis of 51 randomized controlled trials of daily aspirin in prevention of vascular events reported a 25 % reduction in overall cancer incidence from 3 years onward [80]. Interestingly, a reduced risk of developing CRC was also detected during long-term follow-up of healthy women treated with alternate-day 100-mg aspirin dosing versus placebo [81] and of high-risk men treated with a 75-mg controlled-release formulation of aspirin [82] (with negligible systemic bioavailability). Finally, a recent population-based, case-control study including 10,280 adults with an initial diagnosis of CRC and 102,800 adult control participants without CRC showed that the continuous use of low-dose aspirin for 5 or more years was associated with reduced risk for CRC, but overall long-term use that was possibly discontinuous was not [83].

Obesity is associated with a substantial increase of CRC risk in patients with LS; thus, in a prospective study, participants with LS were recruited to the CAPP2 study (in which they were randomly assigned to receive aspirin 600 mg per day or aspirin placebo) to evaluate the association between body mass index and cancer risk. Interestingly, this study showed that this risk is abrogated in those taking aspirin, suggesting that such patients are likely to benefit from obesity prevention and/or regular aspirin [84].

However, from a mechanistic point of view, the most interesting results came from the meta-analyses of CV trials [80, 82, 85], because they suggested that aspirin preventive effect is detectable at daily low doses of 75 mg [84], used for CV prevention [86], and it is saturable at low doses. In fact, at much higher doses (e.g., 1200 mg daily), this effect is not further improved [82].

Several mechanisms of action could explain a chemopreventive effect of high-dose aspirin [74]; among them, the COX-2 inhibition in GI mucosa and its effects on cellular proliferation, apoptosis, and angiogenesis have been proposed [80]. Differently, it seems unlikely that the targeting of nucleated cell could explain the chemopreventive effect of low-dose aspirin due to its pharmacokinetics and pharmacodynamics.

Pharmacodynamics and Pharmacokinetics of Aspirin

Aspirin, as other NSAIDs, reduces prostanoid generation by inhibiting the COX activity [71, 87]. However, aspirin, but not nonaspirin NSAIDs, causes an irreversible inactivation of COX isozymes [86]. Recent evidences have displayed a functional crosstalk between the two monomers of each COX enzyme: both monomers bind the substrate AA, but a monomer acts as an allosteric subunit (regulatory) which transforms the partner monomer into the catalytic one transforming AA into PGG_2; then PGG_2 is transformed to PGH_2 by the peroxidase activity of COX-1 and COX-2. Aspirin binds to one monomer of COX-1 and COX-2 by the interaction with Arg120 residue and modifies covalently COX isozymes by the acetylation of Ser529 and Ser516 on COX-1 and COX-2, respectively; the acetylated monomer becomes the allosteric subunit, and the partner monomer becomes the catalytic monomer. Acetylation of the allosteric subunit of COX-1 and COX-2 by aspirin causes an irreversible inactivation of the COX activity [88]. The acetylated COX-2 has a significantly compromised ability to form PGG_2 but produces an alternative product, 15R-hydroxyeicosatetraenoic acid (15R-HETE) from AA [89]. Studies performed by Smith's group [96] showed that aspirin acetylation of the regulatory monomer of COX-2 is associated with an irreversible inhibition of the catalytic monomer to form PGG_2. In contrast, the

acetylated monomer is able to transform AA into 15R-HETE. Several studies in vitro have shown that 15R-HETE is then metabolized to the epi-lipoxins (LXs) in leukocytes through the action of 5-lipoxygenase (5-LO) [90, 91]; this enzyme is also responsible for initiation of leukotriene biosynthesis. The epi-LXs may cause antiproliferative and anti-inflammatory responses [92–95]. However, convincing evidence that these lipid mediators triggered by aspirin are generated in vivo in humans is lacking.

When orally administered, aspirin is rapidly absorbed in the stomach and upper intestine by passive diffusion across GI membranes [89, 96], peak plasma levels occur 30–40 min after aspirin ingestion, and the functional inhibition of platelets is evident by 1 h. In contrast, it can take up to 6–7 h to reach peak plasma levels after administration of enteric-coated aspirin (100 mg) in healthy subjects [96]. Aspirin has a half-life of 15–20 min [87, 88]. However, despite the rapid clearance of aspirin from the circulation, the inhibitory effect of COX-1 and COX-2 is long lasting because of the irreversible inactivation of the COX isozymes. Thus, in a nucleated cell treated with aspirin, the biosynthesis of prostanoids recovers because a *de novo* protein synthesis of COXs occurs within 3–4 h. In platelets, with a limited capacity of protein synthesis [97], the irreversible inhibition of COX-1 by aspirin persists for the life-span of the platelet [98], and this effect can be reversed only through the generation of new platelets, which in humans have a mean life-span of 8–10 days. Thus, approximately 10–12 % of circulating platelets are replaced within 24 h [87]. This explains the use of aspirin at low doses (75–100 mg/daily) once daily in the antithrombotic therapy, where the target is platelet COX-1. The antiplatelet effect of aspirin is largely independent of systemic bioavailability [99–101] due to the fact that platelet COX-1 is acetylated in the presystemic circulation.

Differently, the use of high dose of aspirin (325–600 mg, given every 4–6 h, and 1.2 g, given every 4–6 h, respectively) is required to obtain an analgesic and anti-inflammatory effect, because these effects mainly occur by inhibiting COX-2 in spinal cord and inflammatory cells [19]. The necessity to use higher doses of aspirin can be explained by the reduced capability of aspirin to inhibit COX-2 than COX-1 and by the reduced plasma concentrations of aspirin, detected in the systemic circulation compared to presystemic compartment, due to its first-pass metabolism [13, 89]. Moreover, the administration of multiple doses is necessary to obtain persistent COX-2 acetylation in nucleated cells that have the capacity to resynthesize the acetylated enzyme within 3–6 days.

Clinical Pharmacology of Aspirin

In vitro experiments show that aspirin is 60-fold more potent to inhibit platelet COX-1 than monocyte COX-2 [13]. When administered in vivo to healthy subjects once daily, aspirin causes a dose-dependent inhibition of platelet COX-1 activity ex vivo, as assessed by the measurement of the generation of TXB_2 [102] in whole blood that is allowed to clot for 1 h at 37 °C (serum TXB_2 is a capacity index of platelet COX-1 activity). However, the maximal inhibition of platelet COX-1 activity is obtained at a low dose of 75–100 mg. At these doses, aspirin inhibits platelet COX-1 activity >95 %, at 1 h after dosing, and this effect persists up to 24 h [102]. The almost complete inhibition of platelet capacity to generate TXA_2 by low-dose aspirin is associated with a profound inhibition of TXA_2-dependent platelet function which persists throughout dosing interval (i.e., 24 h) [98] and represents a fundamental requisite to obtain an antithrombotic effect [87, 88]. In fact, even tiny concentrations of TXA_2 can activate platelets, and they can synergize with low concentrations of other agonists to cause a complete platelet aggregation [103].

The pharmacological effect of aspirin on systemic biosynthesis of TXA_2 is evaluated by measuring the urinary levels of major enzymatic metabolites of TXB_2, such as 11-dehydro-TXB_2, that represent indexes of actual systemic biosynthesis of TXA_2 in vivo, derived mainly from

platelet COX-1 activity [104–106]. However, the assessments of platelet COX-1 activity ex vivo and systemic TXA_2 biosynthesis in vivo are considered indirect biomarkers of aspirin action on COX-1. Between them, a nonlinear relationship has been described; in fact, >97 % inhibition of platelet COX-1 activity ex vivo is required to obtain a reduction of 70–80 % in TXA_2 biosynthesis in vivo [107]. In a recent study performed in healthy subjects treated with enteric-coated low-dose aspirin (EC-aspirin, 100 mg/day) for 7 days, we evaluated the effect of the drug on the extent and duration of platelet COX-1 acetylation at Ser529 by using a novel stable isotope dilution LC-MS/MS (liquid chromatography-mass spectrometry) technique [96]. In this study, the maximal degree of acetylated COX-1 after the seventh dose of the drug averaged 76% and was associated with an average inhibition of platelet COX-1 activity in whole blood of 99% [96]. Thus, in this study, the assessment of platelet COX-1 acetylation at Ser529 has been proposed as direct biomarker of aspirin action on COX-1.

The oral administration of low-dose aspirin once daily is associated with a maximal systemic drug concentration (approximately 7 μM) [99] which may affect only marginally COX-2 activity expressed in nucleated cells. In addition, *de novo* synthesis of the acetylated COX-2 in a nucleated cell may cause a rapid recovery of prostanoid biosynthesis. Thus, the administration of low-dose aspirin did not significantly affect whole blood COX-2 activity ex vivo [108]. Moreover, systemic biosynthesis of vascular PGI_2 (as assessed by the measurement of a major enzymatic urinary metabolite, 2,3-dino-6-keto-$PGF_{1\alpha}$, PGI-M), mainly derived from the activity of COX-2 [33], was only partially affected by low-dose aspirin [109]. However, at higher doses of aspirin, a profound inhibitory effect on the biosynthesis in vivo of PGI_2 was found [109], which might contribute to the apparent reduced antithrombotic benefit detected at high doses of the drug.

The administration of low-dose aspirin doubles the relative risks (RR) of upper GI bleeding (UGIB) in comparison to aspirin nonusers. Since, after oral dosing with low-dose aspirin once daily, the levels of the drug in the systemic circulation are insufficient to cause a substantial inhibition of the biosynthesis of cytoprotective prostanoids in the GI tract, it is plausible that the antiplatelet effect of low-dose aspirin contributes to enhanced incidence of UGIB.

Role of COX-1 in Intestinal Tumorigenesis

Platelet COX-1-Related Mechanisms

As reported above, the implication of platelet activation in cancer is sustained by the analysis of data from long-term follow-up of RCTs of daily aspirin versus control [83, 86]. These trials were designed to determine the efficacy of aspirin in the prevention of vascular occlusive events. However, it was found that regular use of aspirin, even at low doses (which targeted selectively platelets), reduces cancer incidence and mortality, in particular in the GI tract. In addition, one of the CV RCTs in which the chemopreventive effect of aspirin was detected on long-term follow-up (i.e., Thrombosis Prevention Trial, TPT) [110] involved the administration of a controlled-release formulation of aspirin (75 mg) (with negligible systemic bioavailability). Altogether these findings, even if indirectly, support the hypothesis that the inhibitory effect of platelet COX-1 by aspirin is the central mechanism in the anticancer effect of aspirin. As reported above, platelet activation has been found in patients with intestinal cancer [66], and enhanced systemic biosynthesis of TXA_2 is detected in patients with CRC, and it is mainly from platelet COX-1 since it is reduced by low-dose aspirin [67]. Platelets may contribute to tumor development through different molecular mechanisms as reported and explained below.

Inhibition of EGFR and COX-2

Platelets may participate in the early phases of intestinal tumorigenesis through the induction of phenotypic changes in stromal and epithelial cells [111] (Fig. 12.3). In fact, platelets by releasing a

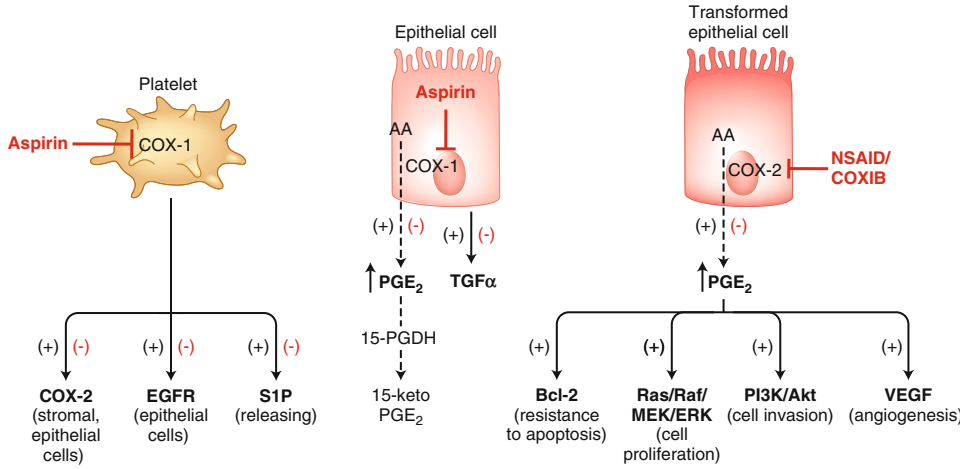

Fig. 12.3 COX-1- and COX-2-related mechanisms in intestinal tumorigenesis. Cyclooxygenase isozymes expressed in different cells (such as platelets, intestinal epithelial cells, and transformed epithelial cells) regulate several pathways involved in tumorigenesis. The chemopreventive effect of aspirin and other NSAIDs (both traditional and selective for COX-2, coxibs) may be achieved by the inhibition of these molecular events. *AA*, arachidonic acid, *PG* prostaglandin, *COX* cyclooxygenase, *NSAIDs* nonsteroidal anti-inflammatory drugs, *coxibs* COX-2 selective inhibitors, *EGFR* epithelial growth factor receptor, *S1p* sphingosine-1-phosphate, *TGF*-α transforming growth factor-α, *15-PGDH* hydroxyprostaglandin dehydrogenase 15-(NAD), *VEGF* vascular endothelial growth factor

plethora of mediators can regulate the expression of COX-2 in stromal cells; the activated stroma, in turn, can release prostanoids and protein mediators, which induce the overexpression of COX-2 in the epithelial cells [95]. The overexpression of COX-2 in the epithelium, through the generation of PGE_2, contributed to the induction of proliferative capacity, migration, invasion, and inhibition of apoptosis of epithelial cells [23]. In this scenario, low-dose aspirin, by the inhibition of platelet activation, may counteract the induction of phenotypic changes in stromal and epithelial cells induced by platelets (Fig. 12.3). In addition novel insights into the mechanism of action of aspirin in preventing CRC, are recently provided. Li and collaborators [112] addressed the hypothesis that the drug normalizes the expression of epidermal growth factor receptor (EGFR), a transmembrane receptor tyrosine kinase of the ErbB family implicated in the etiology of CRC [113] (Fig. 12.3). In FAP patients, the expression levels of EGFR and COX-2 in intestinal epithelial cells resulted to be more abundant with respect to healthy individuals [112]. They found that EGFR and COX-2 proteins were overexpressed in premalignant and malignant lesions and were colocalized. FAP patients, classified as aspirin regular users [two or more standard (325 mg) tablets per week within the previous 12 months], showed lower levels of EGFR and also COX-2 [112]. Based on clinical pharmacology data, the administration of two or more standard (325 mg) aspirin tablets per week used in the study by Li et al. seems to be incompatible with an inhibitory effect of the drug on COX-2-dependent prostanoids produced by nucleated intestinal epithelial cells. In contrast, this aspirin administration schedule might have indirectly downregulated COX-2 expression in colonic epithelial cells through the inhibition of platelet function [1].

Inhibition of Sphingosine-1-Phosphate (S1P) Release

Another mechanism underlying the chemopreventive effect of aspirin in tumorigenesis involved the inhibition of sphingosine-1-phosphate (S1P) release from platelets (Fig. 12.3). Recently, Ulrych and collaborators [114] have

shown that aspirin, both in vitro (at micromolar concentrations) and ex vivo [after dosing with a single analgesic dose (500 mg) or after the administration of an antiplatelet dose of 100 mg day, for 3 days], inhibits the release of S1P from human platelets even after stimulation with the potent peptide agonist of the thrombin receptor protease-activated receptor-1 (PAR-1) [114]. The effect of aspirin was mediated by the inhibition of platelet COX-1-dependent TXA_2 generation. In fact, formation and release of S1P from platelets are dependent on the activation of the TP receptor. S1P plays key roles as regulatory molecule in cancer development [115, 116] by the promotion of cell proliferation, survival, and regulation of angiogenesis, thus suggesting its implication in tumorigenesis. In humans, S1P is a natural constituent of plasma and is generated from sphingosine (SPH) via sphingosine kinase (SPHK), of which two isoforms (SPHK1 and SPHK2) are known [117]. Platelets generate and store high amounts of S1P released upon stimulation with activators of protein kinase C (PKC), such as thrombin and TXA_2 [118]. SPHK is highly active in platelets, which, however, lack the ability to synthesize the substrate SPH [117]. Thus, uptake of extracellular SPH and subsequent phosphorylation to S1P has been proposed as the primary mechanism of S1P formation in platelets [118]. As platelets lack the S1P-degrading enzyme S1P lyase, S1P accumulates intracellularly, and large amounts are released upon platelet activation [118].

Intestinal COX-1-Related Mechanisms

COX-1 is the only isoform constitutively expressed in the normal GI mucosa [126]. The endogenous metabolites of AA formed via COX-1, mainly PGE_2, are involved as local physiological mediator or modulator of mucosal function of the GI tract. In fact, PGE_2 can inhibit acid secretion, stimulate bicarbonate and mucus secretion, as well as affect sodium and chloride ionic flux across the injured mucosa [119].

COX-2 is not detectable in GI epithelial cells under physiological conditions, but it is induced in response to injury and inflammation [19, 120, 121]. COX-2 has been detected in the epithelial cells of colon adenomas and sporadic human colon cancers [113, 122–124], as well as in the stroma of polyps isolated from $APC^{Min/+}$ mice [125].

In serum and tissues, PGE_2 is rapidly metabolized to 15-keto PGE_2 by 15-PGDH, an enzyme which can metabolize a variety of PGs in an NAD+-dependent fashion [126]. 15-keto PGE_2 is further altered by additional enzymatic and nonenzymatic processes to produce 13,14-dihydro-15-keto-PGE_2 and tetranor PGEM. Reduced expression of 15-PGDH leads to prolonged availability and action of PGE_2 and has been linked to several cancers, including colorectal, bladder, pancreatic, and gastric adenocarcinomas [127].

It has been proposed that 15-PGDH downregulation is a very early event in intestinal tumorigenesis occurring even before COX-2 induction [128, 129]. Thus, enhanced PGE_2 might be produced early in colorectal neoplasia through the activity of COX-1 and PGDH downregulation. Recently, it has been shown that the activation of β-catenin signaling, which is deregulated early in colorectal neoplasia, represses PGDH expression [129], leading to increased PGE_2 levels, possibly even before COX-2 upregulation. Enhanced PGE_2 may also contribute to the activation of β-catenin in CRC cells, thus indicating a potential role of PGE_2 in a positive feedback loop [130, 47]. Altogether these results might explain the efficacy of low-dose aspirin to affect early steps in colorectal neoplasia through the inhibition of enhanced COX-1-dependent PGE_2 in colorectal epithelial cells associated with suppression of PGDH expression (Fig. 12.3). Despite this is an interesting hypothesis, it remains to be supported by clinical data. In particular, this hypothesis involves that aspirin, even at low-dose, may affect COX-1 activity by acetylating the protein expressed in the colorectum. Until now this information is missing.

Recently, the relation between the chemopreventive effect of aspirin and the expression of 15-PGDH in the CRC has been described. By analyzing data and samples from Nurses'

Health Study (NHS) and the Health Professionals Follow-Up Study (HPFS), Fink and collaborators found that regular aspirin use was associated with lower risk of CRC that developed within a background of colonic mucosa with high 15-PGDH expression (multivariable HR = 0.49; 95 % CI, 0.34–0.71), but not with low 15-PGDH expression (multivariable HR = 0.90; 95 % CI, 0.63–1.27) (P for heterogeneity = 0.018). Regular aspirin use was associated with lower incidence of CRC arising in association with high 15-PGDH expression, but not with low 15-PGDH expression in normal colon mucosa [131]. This data suggested that 15-PGDH expression level in normal colon mucosa may serve as a biomarker which may predict stronger benefit from aspirin chemoprevention.

Interestingly, the results of these studies suggest the possibility to identify individuals who will get stronger benefit from aspirin chemoprevention. However, the study suffers from several significant limitations that reduce the clinical importance and usefulness of these results. The first limitation is the dose of aspirin. In the NHS, they defined regular aspirin users as women who consumed two or more standard-dose (325 mg) aspirin tablets per week and nonusers as those who reported an intake of a lower number of aspirin tablets per week. In the HPFS, the regular users of aspirin were men who reported consumption of standard-dose (325 mg) aspirin at least two times per week, while men who reported less frequent aspirin consumption were defined as nonusers [131]. Considering the data of pharmacodynamics and pharmacokinetics of aspirin, this dosage regimen and frequency schedule is compatible with the inhibition of platelet COX-1 activity and platelet function, but not with a systemic effect of the drug on COX isozymes expressed in nucleated cells. In addition, the study did not assess direct (acetylation of COX isozymes) or indirect (prostanoid levels in colonic tissue or enzymatic urinary metabolites) markers of aspirin action.

An important issue to address is whether aspirin administration could affect the activity of COX isozymes in the intestinal mucosa. Sample and colleagues [132], using rectal PGE_2 levels as a mucosal biomarker, showed that the administration of different doses of aspirin (81, 325, and 650 mg) for 4 weeks in subjects with prior sporadic colorectal adenomas significantly suppressed PGE_2 levels. In particular, aspirin at 81-mg dose significantly suppressed PGE_2 levels compared to the placebo, as well as at higher doses. Another study performer by Barnes and colleagues [133] showed that aspirin administration reduced two putative surrogate end point biomarkers of chemoprevention of CRC: mucosal PGE_2 formation and transforming growth factor-α (TGF-α) expression (Fig. 12.3). The treatment with aspirin 81 mg daily for 3 months significantly reduced rectal mucosal PGE_2 formation and TGF-α expression in patients with a history of adenomatous polyps. The data obtained in these works indirectly demonstrated that aspirin, administered even at low dose, acts systemically by inhibiting the activity of COX isozymes in the intestinal mucosa. These data should be confirmed in studies where the direct aspirin target will be evaluated, such as the acetylation of serine residues of COXs.

COX-2-Related Mechanisms in Intestinal Tumorigenesis

As reported above, the canonical Wnt pathway activation in the colonic epithelium is a key event in polyp formation and it is associated with the upregulation of several genes involved in tumor development and progression [30], including COX-2 (Fig. 12.3).

Alterations of genetic, epigenetic, and inflammatory pathways involved in intestinal carcinogenesis may influence COX-2 expression leading to elevated prostanoid biosynthesis in tumor microenvironment during the early phases on carcinogenesis. Then, an aberrant expression of COX-2 occurs in epithelial cells and may contribute to the different steps of intestinal tumorigenesis, i.e., hyperplasia and dysplasia to carcinoma and metastasis [134–136]. It is generally well accepted that transcriptional activation of COX-2 can occur early during tumorigenesis.

Due to the complexity of combined genetic alterations and inflammatory signaling occurring in the tumor microenvironment, identifying a single transcriptional pathway which plays a decisive role in promoting constitutive COX-2 expression in colon cancer has been limiting [38].

In normal cells, COX-2 expression levels are largely regulated at the posttranscriptional level through various RNA sequence elements present within the mRNA 3′ untranslated region (3′UTR) of COX-2 mRNA. A well-established mechanism controlling the expression of many inflammatory cytokines, growth factors, and proto-oncogenes is their inherent ability to be targeted for rapid mRNA decay. These cancer-associated gene transcripts are unstable due to the presence of a common *cis*-acting element known as the adenylate- and uridylate (AU)-rich element [137]. AREs mediate their regulatory function through the association of transacting RNA-binding proteins that display high affinity for AREs. It has previously reported that a loss of ARE-mediated regulation is lost early during tumor development. With regard to COX-2 regulation, similar findings have been observed in human colon carcinoma cells [38]. As result of the inability of the COX-2 ARE to function properly in CRC cells, enhanced mRNA stability was detected, and increased expression of a reporter gene containing the COX-2 3′UTR was also observed. To date, at least 16 different RNA-binding proteins have been reported to bind the COX-2 3′UTR [138].

Among them, the mRNA stability factor human antigen R (HuR) [139, 140] overexpression and its cytoplasmatic localization are associated to elevated COX-2 expression that is correlated with advancing stages of malignancy and poor clinical outcome [141, 142].

More recently, small noncoding RNAs called microRNAs (miRNAs) have emerged as global mediators of posttranscriptional gene regulation through their ability to control mRNA stability and translation by imperfect base-pairing to the 3′UTR of its target mRNA [143].

In CRC, differential expression of several miRNAs has been observed, and the loss or overexpression of specific miRNAs can impact various cellular pathways associated with colon tumorigenesis [144, 145]. Currently, 5 miRNAs have been reported to target COX-2 mRNA and control its expression, i.e., miR-16, miR-101, miR-199, miR-143, and miR-542-3p. Since miRNAs can bind imperfectly to the 3′UTR of targeted transcripts to attenuate target gene expression [146], a single miRNA can potentially control a number of putative mRNA targets and impact the expression of a large number of proteins with varying cellular functions. Thus, it is of considerable interest how alterations of miRNA expression in cancer can contribute to tumorigenesis.

COX-Independent Mechanisms of Aspirin

In addition to COX-dependent mechanism (both in platelets and intestinal mucosa), there are several evidences that aspirin may exert a chemopreventive effect through the interference with molecular pathways independent from COX activity. It is noteworthy that most of these antineoplastic effects were found using concentrations of aspirin higher than those detected after dosing with therapeutic doses of the drug.

AMPK and mTOR Signaling

AMP-activated protein kinase (AMPK) is a key energy sensor which regulates cellular metabolism to maintain energy homeostasis. AMPK acts as a metabolic master switch regulating several intracellular systems including the cellular uptake of glucose, the β-oxidation of fatty acids, and the biogenesis of glucose transporter 4 (GLUT4) and mitochondria. The energy-sensing capability of AMPK can be attributed to its ability to detect and react to fluctuations in the AMP/ATP ratio that take place during rest and exercise (muscle stimulation) [147].

Phosphorylated AMPK suppresses the downstream target mTOR (the mammalian target of

rapamycin) which functions as an intracellular nutrient sensor to control protein synthesis, cell growth, and metabolism [148].

Recently, two published works showed that aspirin activates AMPK/mTOR signaling in vitro and in vivo. Hawley and colleagues [149] demonstrated in vitro that at concentrations (millimolar) reached in plasma following administration of salsalate, or aspirin at high doses, salicylate activates AMPK. Salicylate can directly bind AMPK at the same site as the synthetic activator A-769662, to cause allosteric activation and inhibition of dephosphorylation of the activating phosphorylation site, Thr172 [149]. At the same time, Din and coworkers [150] showed that high concentration of aspirin inhibits mTOR signaling in CRC cells as evidenced by inhibition of phosphorylation of downstream effectors of mTOR signaling [i.e., ribosomal protein S6, S6 kinase 1 (S6K1), and eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1)], through the activation of AMPK. Interestingly, they assessed the inhibition of mTOR signaling in healthy subjects treated with aspirin 600 mg/day. After 24 h and 7 days of aspirin intake, the phosphorylation levels of S6 and S6K1 significantly decreased in normal rectal mucosa [150].

NF-kB Signaling Pathway

Several studies have shown that modulation of the NF-kB signal transduction pathway is a key mechanism for the proapoptotic activity of aspirin and other NSAIDs [151, 152]. The NF-kB transcription factor generally exists as a heterodimer bound in the cytoplasm by the inhibitor protein IkB. Following cellular stimulation by specific inducers, IkB is phosphorylated by the IkB kinase (IKK) complex and then degraded by the ubiquitin-proteasome machinery [153]. Subsequently, NF-kB translocates to the nucleus where it regulates transcription of its target genes, including those controlling cell growth. It has been shown that aspirin, as well as sodium salicylate, inhibits IKK-β activity in vitro at millimolar concentration [154] by binding to IKK-β, thus competing with ATP for the binding to the kinase, an event necessary to phosphorylate IkB.

Wnt/β-Catenin Pathway

The aberrant activation of the Wnt/β-catenin pathway is the first step in almost all CRC. The consequence of this is the accumulation of the β-catenin in the cytoplasm which can translocate to the nucleus where it binds with members of the T-cell factor (Tcf)/lymphoid enhancer factor (Lef) family of transcription factors and activates the transcription of target genes such as cyclin D, c-Myc, and COX-2 [155]. Bos and colleagues [156] showed that aspirin caused a time- and concentration-dependent increase in β-catenin phosphorylation, thereby reducing Wnt/β-catenin pathway activity, in CRC cell lines. They found that aspirin acted through the inhibition of protein phosphatase activity, the enzyme involved in the regulation of the phosphorylation status of β-catenin.

ERK Signaling

The extracellular signal-regulated kinase (ERK) signaling pathway is a major determinant in the control of different cellular processes such as proliferation, survival, differentiation, and motility. This signaling resulted to be hyperactivated in a high percentage of tumors [157]. Because of its multiple roles in the acquisition of a complex malignant phenotype, specific blockade of the ERK pathway is expected to result in an antiproliferative effect but also in antimetastatic and antiangiogenic effects in tumor cells [157]. It has been shown that aspirin and other NSAIDs inhibit ERK signaling by preventing the binding of Ras oncogene to c-Raf kinase in vitro [158].

Inhibition of AP-1 Activity

AP-1 is an inducible transcription factor containing products of the *jun* and *fos* oncogene

families [159, 160]. AP-1 is activated in response to a number of stimulants including the tumor promoter phorbol esters, epidermal growth factor, tumor necrosis factor-α, and interleukin-1 [159]. Some of the genes known to be regulated by AP-1 are involved in the immune and inflammatory responses, tumor promotion, and tumor progression. Dong and colleagues [161] evaluated the antitumor effect of aspirin in JB6 cells, a well-developed cell culture model for studying tumor promotion. They found that aspirin and salicylate, at millimolar concentration, inhibit transcription factor AP-1 activity and tumor promoter-induced transformation through a mechanism independent of PG synthesis and of the inhibition of Erk1 and Erk2 pathways, but probably involving the intracellular H^+ concentration.

Acetylation of Non-COX Proteins by Aspirin

Studies over the past decades suggest that, besides COXs, aspirin acetylates other cellular proteins. Experiments with radiolabeled ^3H or ^{14}C aspirin demonstrated that aspirin acetylates several proteins in vitro and in vivo through a transacetylation reaction [162, 163]. Aspirin acetylates human serum albumin and fibrinogen in vitro and in vivo [164, 165]. It can also acetylates several other proteins and biomolecules, such as hemoglobin, DNA, RNA, histones, and transglutaminase, as well as other plasma constituents including hormones and enzymes [162, 166]. Recently, it has been shown that aspirin, at micromolar concentration, acetylates the tumor suppressor protein p53, a known regulator of apoptosis, in human breast cancer cells. This event was associated with the induction of p21CIP, a protein involved in cell cycle arrest, and Bax, a proapoptotic protein [167]. Thus, aspirin could exert its anticancer effects by involving the acetylation of the tumor suppressor p53 and the induction of p21CIP.

Other Antiplatelet Therapies in Cancer

Several evidences support the hypothesis that platelet activation is involved in the development of cancer, particularly CRC and in facilitating metastasis [168]. In this setting, the development of platelet inhibitors that influence malignancy progression and clinical testing of currently available antiplatelet drugs represents a promising area of targeted cancer therapy.

At this time, a limited number of mechanistic studies and clinical trial data support the use of currently available antiplatelet agents in cancer therapy and the combination of antiplatelet therapy with existing tumor-targeted therapy. In contrast, the laboratory data using antiplatelet therapy continue to accumulate.

Blockage of Platelet GPIIb/IIIa Receptors

The importance of platelet receptor GPIIb/IIIa in the tumor mechanisms was showed by Boukerche and collaborators who demonstrated that human malignant melanoma cells directly interact with platelets through the GPIIb/IIIa receptor and cause platelet aggregation [169]. In this study, Fab fragments of a monoclonal antibody MoAb (LYP18), directed against the platelet GPIIb-IIIa complex, inhibited platelet-melanoma interactions and platelet-platelet aggregation. In a murine model of metastasis, Nierodzik and colleagues founded that the blockage of the platelet GPIIb/IIIa receptor using the monoclonal antibody 10E5 decreased lung colonization of cancer cells [170]. A challenging aspect of the administration of GPIIb/IIIa antagonists in the clinical setting has been the need for intravenous administration of these agents, which are now widely used in high-risk acute coronary syndromes. However, an oral inhibitor of GPIIb/IIIa, XV454, has halted experimental metastasis formation in a murine model of lung cancer [171].

Blockage of Platelet GPVI Receptor

Glycoprotein (GP)VI is a key receptor for collagen on the platelet surface. It is a member of the immunoglobulin superfamily and is uniquely expressed on the surface of platelets, where it is assembled with the immunoreceptor tyrosine activation motif subunit, FcR-γ. Jain and colleagues have shown that in a murine model of metastasis, using either a Lewis lung carcinoma (D121) or melanoma (B16F10.1) cell line, an approximately 50 % reduction in the number of visible tumor foci in GPVI-deficient mice as compared with control C57BL/6 J mice was observed [172].

In vitro data suggest that also a GPVI antagonist (revacept) may affect metastasis by inhibiting platelet-tumor cell crosstalk [173]. Revacept can bind to atherosclerotic endothelium both with and without plaque rupture; it binds vascular collagen, and thus it might interfere with other collagen-dependent pathways including α2/β1 integrins or vWF-mediated GPIb activation [174]. Revacept reduces platelet adhesion and aggregation without increasing the risk of bleeding complications and without affecting the general hemostasis [174].

Dovizio et al. have recently shown a novel pharmacological effect of revacept in platelet-cancer cell crosstalk [173]. In fact, this drug was able to interfere with the interaction of platelet collagen receptors with galectin-3 expressed in colon cancer cells HT-29. Thus, the drug, at clinically relevant concentrations [174], completely prevented the platelet-induced upregulation of COX-2 in HT-29 cells and the induction of epithelial-mesenchymal transition (EMT) markers which occurred in tumor cells by platelet interaction [173].

P-Selectin Antagonists

P-selectin is a cell surface adhesion molecule that has a central role in mediating interactions between platelets and both leukocytes and the endothelium [175]. When P-selectin is expressed on activated platelets and endothelial cells, its primary ligand, P-selectin glycoprotein ligand type 1 (PSGL-1), mediates the initial tethering and rolling process that precedes leukocyte transmigration through the vessel wall [176]. It has been proposed that P-selectin facilitated the interaction between tumor cells with both platelets and endothelial cells via sialylated fucosylated carbohydrates [177]. In addition, it has been shown that P-selectin facilitates human carcinoma metastasis in immunodeficient mice by mediating early interactions of platelets with blood-borne tumor cells via their cell surface mucins, and this process can be blocked by heparin [178].

PAR Antagonists

Thrombin, a serine protease generated by the coagulation cascade, is responsible for the generation of fibrin and in addition is a potent activator of human platelets via actions on two platelet surface G-protein-coupled receptors, PAR-1 and PAR-4 [179]. Italiano and collaborators showed that distinct populations of platelet α-granules, containing different angiogenesis influencing proteins, can be differentially released. The release of a different set of α-granules from platelets is regulated by PAR-1 and PAR-4 activation [180]. PAR-1 and PAR-4 have been shown to regulate the release of endostatin and VEGF from human platelets. These protease-activated receptors could therefore play a crucial role in regulating angiogenesis and in turn could regulate the processes of wound healing and tumor growth [181]. In a murine model of hematogenous metastasis, melanoma cells were intravenously injected in PAR-4 KO mice, and protection from lung metastases was observed. Thus, this study suggests that thrombin-induced platelet activation makes an important contribution to hematogenous metastasis [181].

P2Y12 Receptor Antagonists

Once released, ADP causes the coordinate activation of G-protein-coupled receptors, the purinergic receptors [88, 182, 183]. There are 3 types of receptors for ADP on platelets: a P2X-type ion channel-linked receptor and 2 P2Y-type G-protein-coupled receptors, P2Y1 and P2Y12 [182]. Activation of P2Y1 receptors induces a phospholipase C-mediated increase of intracellular calcium leading to platelet shape change and initial reversible aggregation via Gαq. P2Y12 receptors in their turn complete the aggregation response initiated by P2Y1receptors via Gαi-mediated inhibition of adenylyl cyclase and through a less well-defined activation of PI3K [184]. The effects of other antiplatelet agents, such as the antagonists of P2Y12 receptor, in the prevention of cancer and tumor metastasis remain to be characterized in humans. However, several experimental evidences sustain a possible anticancer effect of these agents. It has been reported that activated platelets promote metastasis through the release of small molecules such as ATP and ADP [185]. As platelet activation is largely mediated through ADP engagement of the purinergic receptor P2Y12 on platelets, P2Y12 represents an attractive target for inhibiting tumor metastases. It has been shown that the thienopyridine SR 25989, an enantiomer of the anti-aggregant clopidogrel (Plavix) lacking anti-aggregant activity, inhibits endothelial cell proliferation in vitro by increasing the expression of endogenous thrombospondin-1, a natural potent inhibitor of angiogenesis. The antiangiogenic effect of SR 25989 was further assessed in vitro in a quantitative assay of angiogenesis using a fragment of rat aorta embedded in a fibrin gel and in vivo, using a pulmonary metastatic model in C57BL/6 mice inoculated in the foot pad with the highly metastatic melanoma cell line B16 F10 [186]. Recently, Wang et al. demonstrated that tumor metastases are reduced in P2Y12-deficient mice [187]. The coadministration of the antiplatelet drugs aspirin and clopidogrel (an antagonist of P2Y12 receptor) prevents or delays hepatocarcinoma and improves survival in a mouse model of chronic immune-mediated hepatitis B [188].

Conclusive Remarks

Several evidences support the anticarcinogenic effect of aspirin and other NSAIDs [1, 120]. The findings that the protective effect of aspirin against cancer, particularly CRC, does not appear to be dose dependent and the maximal effect is detected at low doses—which are the same recommended for the prevention of CV disease—strongly support the hypothesis that the inhibition of platelet function is an important determinant [1, 13, 120, 189]. Platelets are considered inflammatory cells [190], and when activated they release a massive quantity of a wide spectrum of growth and angiogenic factors, inflammatory proteins, lipids, and vesicles containing also genetic material, including miRNAs. Platelets may be activated as a consequence of vascular and epithelial damage, as it may occur in response to lifestyle and environment factors. Altogether these events alter the normal functions of epithelial cells, thus leading to cellular transformation through the overexpression of COX-2 [95, 191]. Thus, antiplatelet agents may play a role in the prevention of CRC by modifying epigenetic mechanisms involved in colorectal tumorigenesis. Platelets may also contribute to the progression of cancer through the development of metastasis [168, 173, 192, 193]. Several mechanisms have been described, including the formation of platelet aggregates surrounding tumor cells which may support tumor cell survival and protection from immune elimination and enhancement of the adhesion of tumor cells to the endothelium, thus leading to tumor cell arrest and extravasation. The recent findings showing that platelet-derived signals induce the activation of programs [173, 192] provide new insights into the molecular mechanisms which modulate the plasticity of cellular phenotypes and open the way to novel

therapeutic interventions using antiplatelet agents to restrain and possibly prevent the development of cancer metastasis. Additional mechanistic studies to test the "platelet hypothesis" should be performed in animal models of intestinal cancer and, ideally, in different stages of the human disease. These could help to address the current uncertainty concerning the optimal chemopreventive dose and dosing regimen of aspirin. If this hypothesis would be confirmed by ongoing studies, this would provide a rationale for targeting other pathways of platelet activation and assessing the efficacy and safety of combined antiplatelet strategies for cancer prevention.

An important field of clinical research is focused on the discovery of biomarkers to select the individuals who will respond better to the antineoplastic effect of aspirin. They include plasma inflammatory markers, such as soluble tumor necrosis factor receptor-2 (TNF-R2), as well as the tumor expression levels of genes involved in prostanoid biosynthesis, including COX-2, or signaling pathways implicated in the aberrant expression of COX-2, such as PI3K. In particular, Liao and colleagues have highlighted the benefit of aspirin use in a molecular-defined subgroup of patients affected by metastatic CRC who carried activating mutations in PIK3CA (phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha) [194]. Most of these studies have the limitation of being large cohorts of participants who provided data on aspirin use from a questionnaire. Thus, these results should be confirmed in large RCTs. In these clinical studies, the use the innovative systems biology approach for the analysis of heterogeneous data sets (genomics, epigenomics, proteomics, lipidomics, and clinical) would allow to perform dynamic systems modeling of candidate pathways involved in the antineoplastic efficacy of aspirin. This strategy will also allow to identify CRC susceptibility profiles and to use them to develop new biomarkers to predict the occurrence/recurrence of CRC.

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